

Article

Assisted Stacking of Fungal Disease Resistance Genes in Central American Coffee Cultivars

Eduardo Granados Brenes ¹, Laércio Zambolim ², Dênia Pires de Almeida ², Poliane Marcelle Ribeiro ³,
Bruna Lopes Mariz ² and Eveline Teixeira Caixeta ^{2,4,*}

¹ Department of Phytopathology, University of Costa Rica, Sede del Atlántico, Turrialba 30501, Costa Rica; eduardo.granados@ucr.ac.cr

² Institute of Biotechnology Applied to Agriculture, Biocafé, Federal University of Viçosa, Viçosa 36570-900, MG, Brazil; zambolim@ufv.br (L.Z.); denia.almeida@ufv.br (D.P.d.A.); bruna.mariz@ufv.br (B.L.M.)

³ EMATER—Technical Assistance and Rural Extension Company—Regional, Viçosa 36570-900, MG, Brazil; poliane.cardoso@emater.mg.gov.br

⁴ Brazilian Agricultural Research Corporation, Embrapa Café, Brasília 70770-901, DF, Brazil

* Correspondence: eveline.caixeta@embrapa.br; Tel.: +55-(31)-3612-2436

Abstract: The main diseases that affect coffee production worldwide are coffee leaf rust (CLR) and coffee berry disease (CBD), caused by fungi *Hemileia vastatrix* and *Colletotrichum kahawae*, respectively. The identification of cultivars with stacking resistance genes is of paramount importance for the control of these diseases. This work aimed to profile the phenotypic and genetic resistance of 160 genotypes belonging to 36 commercial coffee cultivars from five Central American countries regarding resistance to races II and XXXIII of *H. vastatrix* through phenotypic evaluation and evaluations associated with the genetic loci of resistance to CLR and CBD by molecular markers. Of the 160 genotypes from Central America evaluated, 26.25% presented genes stacked to the three loci of resistance to CLR and the locus of resistance to CBD, and resistance to races II and XXXIII when inoculated with urediniospores. In addition, 14 genotypes were identified with the presence of the *S_H3* gene, whose resistance has not yet been broken. This work revealed errors in passport data or hybridizations in cultivars and even possible resistance breakdown in the Catimor genetic group. These results are essential to the search for strategies in coffee genetic breeding programs.

Keywords: *Coffea arabica*; multiple resistance; pyramided gene; molecular markers



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1. Introduction

Coffee originates from Africa, specifically from the regions of Ethiopia from which the crop was taken to the rest of the world. One of the major challenges for coffee growers worldwide is the natural occurrence of coffee leaf rust (CLR) epidemics caused by the fungus *Hemileia vastatrix*, due to its ease of dissemination and adaptation to the geographic locations of coffee production [1]. CLR is considered the most significant coffee diseases, with estimated costs to the coffee industry of \$1–2 billion annually [2,3]. Coffee production in Central America has been challenging over the past two decades. In the years 2008–2013, an epidemic event was recorded that caused great losses to coffee growers in the region, generating significant losses of 16% in Central America and 16% in Colombia [3]. Recently, the presence of this pathogen has been identified in coffee-growing regions that previously did not record the disease, such as China, Hawaii, Saudi Arabia, and Peru [4–7].

The occurrence of the fungus in several producing regions and the great variability of the pathogen has triggered the emergence of more complex races of *H. vastatrix* in

search of adaptability to climatic conditions that favor the pathogen, causing a loss of resistance in cultivars that were previously considered resistant [8]. Many of these new breeds carry virulent genes capable of overcoming the resistance of cultivars containing resistance genes [9]. The increase in disease in crops and the presence of new races or pathotypes lead to the increased use of fungicides to control the disease, such as copper, triazoles, strobilurins, among other molecules [8]. However, the indiscriminate use of these products can cause environmental damage, and the fungus loses its sensitivity to the molecules, although this phenomenon has not yet been scientifically confirmed, reducing its effectiveness in controlling the disease.

In view of this scenario, researchers have intensified the identification and development of new cultivars with resistance to CLR. Resistance to coffee rust is conferred by at least 11 characterized genes: S_H1 , S_H2 , S_H4 , and S_H5 in *Coffea arabica*; S_H3 in *Coffea liberica*; and S_H6 to S_H9 have been identified in *Coffea canephora* [10,11]. The difference in ploidy is the factor that makes it impossible to crossbreed these species to incorporate resistance. Coffee plants called Híbrido de Timor (HdT) correspond to natural hybrids between *C. arabica* and *C. canephora* and have different combinations of the genes S_H6 , S_H7 , S_H8 , S_H9 , and S_H10 , in addition to others not yet characterized [12]. In addition to resistance to rust, these coffee plants carry genes for resistance to other diseases, in addition to being explored for the development of cultivars with increased production potential and beverage quality.

Another disease of great relevance in coffee growing is anthracnose of the fruits, Coffee Berry Disease (CBD), caused by the fungus *Colletotrichum kahawae*. This disease is only found in Africa, but there is concern about its spread throughout the world. Its arrival in the Americas could cause the collapse of global coffee growing. In Africa, CBD has been causing significant yield losses (40 to 80%) in susceptible varieties. No other coffee disease has had as profound an economic impact as CBD in Africa, which has contributed to a sharp decline in total arabica coffee exports from the continent. The estimated economic loss of exportable *C. arabica* beans due to CBD in Africa is between \$300 to 500 million per year [13].

Therefore, the identification of materials that contain genes that confer resistance as a form of preventive breeding is important. Ref. [14] showed CBD resistance to be governed by a T gene in HdT and Catimor, R, and K genes in Rume Sudan and a K gene in K7, identified as the *Ck-1* gene in Catimor-derived individuals and this gene is suggested to corresponded to the T locus previously identified in HdT.

Breeding programs are interested in introgressing genes from different species into the *C. arabica* germplasm by hybridization. A widely used tool is the isotype of molecular markers. To support breeding for rust resistance, a genetic map of *C. arabica* was constructed, which allowed the identification of two regions (QTLs—Quantitative Trait Loci) that correspond to two major dominant and independent genes that confer resistance to three pathotypes of *H. vastatrix*, race I, race II, and pathotype 001 [15]. From this mapping, molecular markers flanking these two loci were developed and obtained, one being a sequence-characterized amplified region (SCAR) marker (CaRHv8) and a microsatellite (SSR016) associated with one of the QTLs and also a SCAR (CaRHv9) and a cleaved amplified polymorphic sequence (CAPS) (CaRHv10_CAPS) associated with another QTL [16]. These identified loci/QTLs come from the HdT (accession HdT-UFV 443-03), one of the main sources of resistance to coffee rust. A *CC-NBS-LRR* gene family for resistance to *H. vastatrix* has been identified in this same resistance source, but in another accession (HdT CIFIC 832/2), and a molecular marker called CARF005 has been developed [12,17].

Working with other sources of rust resistance, such as the Indian selection S.288, ref. [18] mapped a major resistance gene using three SCAR markers (BA-48-21-f, BA-124-12K-f and SP-M16 S_H3) and one SSR marker (Sat244). This mapped gene corresponds to the

S_H3 gene originated from the species *C. liberica* and introgressed into *C. arabica* by natural crossing that gave rise to the Indian selections.

Molecular markers have also been identified for the resistance gene to another important coffee disease, CBD. Ref. [14] characterized the gene called *Ck-1*, from the resistance source HdT, using a genetic mapping approach. In this work, two SSR and eight AFLP markers were identified as being linked to the *Ck-1* gene. Molecular marker-assisted selection (MAS) is an important tool that can detect and analyze the genetic polymorphisms present in several species, identifying high resistance to pathogens restricted to the species [19].

The introduction of multiple genes into a cultivar in genetic breeding is called gene pyramiding. This breeding strategy is important for obtaining broad-spectrum and long-lasting resistance, as well as conferring multiple resistance [16,20]. In this context, molecular markers can effectively assist in the identification of genotypes that have multiple resistance genes in the presence of a dominant or epistatic effect, allowing for the pyramiding of several resistance genes in a single cultivar [16,21].

Molecular marker-assisted selection (MAS) is an important tool, not only to identify, monitor, and pyramid genes of interest in breeding, but it also can detect and analyze genetic polymorphisms present among genotypes [18,21]. For disease resistance breeding programs to be successful, it is also necessary to continually incorporate new sources of resistance. These sources can be identified by inoculating different plant accessions with different races of the pathogen, highlighting the most frequent races that present different levels of aggressiveness. In the coffee–*H. vastatrix* pathosystem, race II is most frequently found in Brazil [9] and in other coffee-producing countries in the American continent [8]. This race presents the *v5* virulence gene and its widespread occurrence is probably due to the planting, in various regions, of coffee cultivars that contain only the *S_H5* gene [9].

From the use of resistant cultivars containing more resistance genes, in the Americas, new races have been identified [8]. Race XXXIII was identified in Brazil, supplanting coffee genotypes considered resistant to rust in coffee plants of the Catimor genetic group. This is a more complex breed and has the virulence genes *v5* and *v7* or *v5*, *v7*, and *v9* [22]. The inoculation of coffee plants with these two races, the most frequent and the most virulent, is of utmost importance not only in the search for new sources of resistance but also in order to study the potential for resistance or susceptibility of coffee plants that are currently planted in the Americas.

In view of the above, the objective of this study was to characterize cultivars of the different countries in Central America through inoculations with different races of *H. vastatrix* and the use of molecular markers associated with different loci/genes of resistance to this pathogen. The cultivars were also analyzed for the presence of the *Ck-1* gene that confers resistance to *C. kahawae* to identify promising cultivars that may also confer resistance to this disease that is absent in the Americas.

2. Materials and Methods

The plant material used was obtained through partnerships between researchers from Brazil and Central American countries. Thirty-six coffee cultivars from different Central American countries were obtained. One to five plants of each cultivar were evaluated, totaling 160 genotypes (Table 1). Among the 36 cultivars, cultivars belonging to the Typica–Bourbon genetic group were obtained, and coffee growing in Latin and Central America is predominantly performed with cultivars from this group, due to the historical process of the introduction of these plants. In addition to this genetic group, cultivars belonging to genetic groups such as Catimor, Sarchimor, and Catuaí x Híbrido de Timor were also obtained, originating from crosses with HdT, due to their multiple resistance to rust and

CBD. From these germplasms, 160 seeds of cultivars were sown in individual plastic pots of 3 L in a greenhouse to obtain seedlings. The plants were kept in a greenhouse with temperatures ranging from 20 °C to 28 °C (20°45'28.69" S, 42°52'11.47" W, 648 m altitude).

Table 1. Arabica coffee cultivars for characterization of genetic resistance to CLR and CBD, from Central America.

Country	Cultivar	Parents	Genetic Group	Number of Genotypes
Costa Rica	Costa Rica 95	Híbrido de Timor 832/1 × Caturra	Catimor	1
	San Izidro 48	Villa Sarchi × Híbrido de Timor	Sarchimor	6
El Salvador	Bourbon	Bourbon	Bourbon–Typica	6
	CR95_Izalco	Híbrido de Timor 832/1 × Caturra	Catimor	6
	Cuscatleco_1	Híbrido de Timor × Villa Sarchi T5296	Sarchimor	6
	Cuscatleco_2	Híbrido de Timor × Villa Sarchi T5296	Sarchimor	6
	Pacamara_1	Pacas × Maragogipe Vermelho (Mutaç�o Typica)	Bourbon–Typica	5
	Pacamara_2	Pacas × Maragogipe Vermelho (Mutaç�o Typica)	Bourbon–Typica	2
	Pacamara_3	Pacas × Maragogipe Vermelho (Mutaç�o Typica)	Bourbon–Typica	1
	Pacas_1	Mutaç�o Bourbon	Bourbon–Typica	1
	Pacas_2	Mutaç�o Bourbon	Bourbon–Typica	4
	San_Pacho	Caturra × San Bernardo (Mutaç�o Typica)	Bourbon–Typica	1
	San_Pacho_1	Caturra × San Bernardo (Mutaç�o Typica)	Bourbon–Typica	4
	San_Pacho_2	Caturra × San Bernardo (Mutaç�o Typica)	Bourbon–Typica	6
	San_Pacho_3	Caturra × San Bernardo (Mutaç�o Typica)	Bourbon–Typica	2
Guatemala	Anacaf�	(Catimor T-5175 × Caturra) × Pacamara	Catimor	6
	Catigua_MG2	Catua� Amarelo IAC 86 × H�brido de Timor UFV440-10	H�brido de Timor	6
	Catimor_1	Catimor T-5175	Catimor	2
	Catimor_2	Catimor T-5175	Catimor	6
	Catimor_5175	Catimor T-5175	Catimor	2
	Colismor	Pache Colis × Catimor T-5175	Catimor	4
	Guapa	Catua� × Catimor	Catimor	6
	Sarchimor	H�brido de Timor CIFC 832/2 × Villa Sarchi	Sarchimor	6
Honduras	Catimor_8667	Catimor	Catimor	6
	Catimor_T5175	H�brido de Timor 832/1 × Caturra	Catimor	6
	Centroamericano_F2	Sarchimor T5296 × R�me Sudan	Sarchimor	6
	Centroamericano_F3	Sarchimor T5296 × R�me Sudan	Sarchimor	6
	Geisha	Geisha	Typica	3
	IHCAFE_90	H�brido de Timor 832/1 × Caturra	Catimor	6
	Lempira_1	H�brido de Timor 832/1 × Caturra	Catimor	6
	Lempira_2	H�brido de Timor 832/1 × Caturra	Catimor	6
	Pacamara_4	Pacas × Maragogipe Vermelho (Mutaç�o Typica)	Bourbon–Typica	6
	Parainema	T5296	Sarchimor	2
	Parainema_Apical	T5296	Sarchimor	1
Dominican Republic	Catimor_3	-	Catimor	6
	Catimor_4	-	Catimor	5

×: represents crossing between genotypes.

When the seedlings formed fully expanded leaves, they were collected for analysis of genetic resistance via inoculation with races II and XXX III of *H. vastatrix* and DNA extraction for analysis by molecular markers associated with resistance to CLR and CBD.

2.1. Multiplication of Urediniospores of *Hemileia vastatrix*

The urediniospores of races II and XXXIII of *H. vastatrix* were obtained from the BioCaf -UFV collection, where they are preserved in an ultrafreezer at −80 °C. The uredin-

iospore germination tests before inoculation were performed on 1% water agar plates [22]. The plates were placed in a dark chamber for 24 h at a temperature of 22 °C. Germinated and non-germinated spores were counted using a stereoscopic microscope, considering those with a germ tube twice the diameter of the urediniospore as germinated and viable. Only urediniospores with germination above 30% were used.

The multiplication of urediniospores of *H. vastatrix* was carried out in seedlings of the cultivar caturra, due to its high susceptibility to rust. A total of 50 mg of urediniospores of race II (v5) characterized by [23] and race XXXIII (v5,7 or v5,7,9) characterized by [22] from the Biocafé collection, based at the UFV, were used.

Inoculation was performed using a brush on the leaves of coffee seedlings and placed in a dark chamber. Forty-eight hours after incubation in a fog chamber (at a temperature of 22 °C and with relative humidity close to 100%), the plants were transferred to an incubation chamber with a temperature of 22 °C, where they remained for 30 days. Urediniospores were collected and stored in a desiccator at 5 °C, with a relative humidity of 50%, following the protocol by [24].

2.2. Preparation and Inoculation of *H. vastatrix* Urediniospores on Coffee Leaf Disks

Leaves of the 160 genotypes of Central America and cultivar Caturra, susceptible to rust (positive control), were used to obtain 16 disks with 2 cm in diameter of plant tissue for the inoculation tests. The experiment was repeated twice in time, the first in February 2021 and the second in November of the same year.

Inoculation was performed by spreading 1 mg of urediniospores using a brush on the abaxial surface of each coffee leaf disk kept on a stainless-steel screen in a germination box (plastic box 11 × 11 × 3 cm), and then moistened with sterilized distilled water [25]. The germination boxes were closed and maintained in the dark for 48 h at 22 °C and then transferred to a chamber at a temperature of 22 °C and relative humidity close to 100%, under a 12 h light photoperiod. The disks were cleaned 48 h after inoculation with cotton moistened in sterilized distilled water to eliminate excess urediniospores on the abaxial surface, taking care not to cause damage, to avoid the presence of parasitic fungi of *H. vastatrix* [22]. The germination box was transferred to an incubation chamber with a temperature of 22 °C, where they remained for 45 days for evaluation.

2.3. Phytopathological Parameters of Genetic Resistance Linked to *H. vastatrix*

In search of qualitative responses associated with resistance to the *H. vastatrix* races evaluated, for 16 leaf disks in each germination box, the phenotyping proposed by [26] was performed with the parameters: incubation period (IP) (the period from inoculation until 50% of the disk is observed with CLR symptoms); latent period (LP) (the period from the tenth day after inoculation until the observation of 50% of the final incidence of disks with sporulating pustules); incidence (IN) (the final percentage of the disks showing CLR symptoms); urediniospore production (UP) (the final quantity of urediniospores produced, using the methodology proposed by [27]); the area under the disease progress curve (AACPD) (which was estimated based on the severity data at the end of the experiment, taking into account the average progression of symptoms by adding the evaluations over 45 days, as this is a study of inference evaluations of the quantitative resistance of two cultivars, it was calculated, as per [28]; and severity (SE) (rust severity was assessed on 16 leaf disks in each germination box; it was measured according to the percentage of leaf disk area infected). The analysis was performed every two days from the tenth day after inoculation, up to a total of 45 days evaluated.

The required rust severity scale was described by [29] with modifications, in which the scores indicate the area of the disks with a presence of urediniospores: score 0 (0%);

score 1 ($\leq 1\%$), score 2 ($\leq 10\%$); score 3 ($\geq 10\%$ and $\leq 15\%$), score 4 ($\geq 15\%$ and $\leq 30\%$), score 5 ($\geq 30\%$ and $\leq 50\%$), and score 6 ($\geq 50\%$) (Figure 1). The phenotyping of phytopathological parameters had two replicates in time. The experiment was conducted in a completely randomized manner. The comparison of media was at 5% significance by InfoStat [30]. Each of the 16 disks that make up the box was considered to be a repetition of each cultivar.

Severity score						
0	1	2	3	4	5	6
						
(0%) leaf disc area with sporulation	($\leq 1\%$) leaf disc area with sporulation	($\leq 10\%$) leaf disc area with sporulation	($\geq 10\%$ and $\leq 15\%$) leaf disc area with sporulation	($\geq 15\%$ and $\leq 30\%$) leaf disc area with sporulation	($\geq 30\%$ and $\leq 50\%$) leaf disc area with sporulation	($\geq 50\%$) leaf disc area with sporulation

Figure 1. Scale of severity of infection by *H. vastatrix* urediniospores. Score 0—leaf disk with area of sporulation of 0%, score 1—leaf disk with area of sporulation ($\leq 1\%$), score 2—leaf disk with area of sporulation ($\leq 10\%$), score 3—leaf disk with area of sporulation ($\geq 10\%$ and $\leq 15\%$), score 4—leaf disk with area of sporulation ($\geq 15\%$ and $\leq 30\%$), score 5—leaf disk with area of sporulation ($\geq 30\%$ and $\leq 50\%$), score 6—leaf disk with area of sporulation $\geq 50\%$.

2.4. Molecular Characterization of Resistance/Susceptibility to *H. vastatrix* and *C. kahawae*

All accessions from Central America were subjected to genomic DNA extraction, according to the methodology proposed by [31]. Five loci linked to resistance were analyzed, using eight molecular markers adopted in the scientific community for their efficiency in CLR and CBD characterization.

2.4.1. Molecular Markers Associated with the Coffee Resistance gene S_{H3} —Locus A

To identify genotypes carrying alleles of the S_{H3} gene for resistance to *H. vastatrix*, the amplification of the extracted DNA samples was performed using two markers, SCAR Ba-124-12K-f and the SSR marker Sat 244 linked to this identified gene [18].

Individuals with a presence of dominant alleles amplified by the two molecular markers linked to the gene were considered carriers of the S_{H3} gene. Five controls were used, of which three genotypes carried the resistance allele to the S_{H3} gene (CIFC H147/1, CIFC H153/2 and S.288/23) and two susceptible genotypes without S_{H3} (Caturra Vermelho—CIFC 19/1 and Catuaí amarelo IAC 64-UFV 2148/57). PCR amplifications were performed in a final volume of 25 μ L, containing 50 ng of DNA, 1X PCR reaction buffer, 2.0 mM MgCl₂, 0.1 mM dNTP, 0.4 μ M of forward and reverse primers, and 0.5 units of Taq DNA polymerase. All reactions were performed in the PTC-200 (MJ Research) and Veriti (Applied Biosystems) thermocyclers, with an initial denaturation phase at 95 °C for 5 min; 35 cycles at 94 °C for 45 s, annealing temperatures of 52 °C for Sat244 and 56 °C for Ba-124-12KF for 45 s, followed by an extension at 72 °C for 45 s and a final extension at 72 °C for 10 min. The products resulting from the PCR reaction were separated by electrophoresis in a 6% denaturing polyacrylamide gel and visualized by staining with silver nitrate [32].

2.4.2. Molecular Markers Associated with Resistance Loci/QTLs—Loci B and C

To characterize the genotypes regarding resistance to races I and II and pathotype 001 of *H. vastatrix*, the molecular markers SSR 016 [33], CaRHv8, and CaRHv9 [16] were linked to loci/QTLs.

The markers SSR16 and CaRHv8 are linked to the locus/QTL of linkage group 2. SSR16 presents a codominant pattern, identifying individuals BB, Bb, and bb. CaRHv8 is a dominant marker, linked in repulsion, marking only the recessive allele *_b*; therefore, the

presence of the allele allows for the identification of Bb or bb genotypes, and the absence of the allele BB genotypes [16]. The marker CaRHv9 is linked to the locus/QTL of linkage group 5 and behaves as a dominant and coupled marker, identifying individuals with genotypes C₋ and cc in the population [15]. The HdT UFV 443-03 was used as resistant control, and the Catuaí Amarelo IAC 64 (UFV 2148/57) as susceptible.

PCR amplification for CaRHv8 and CaRHv9 was performed in a final volume of 20 µL, containing 50 ng of DNA, 1X PCR reaction buffer, 2.0 mM MgCl₂, 0.15 mM of each dNTP, 0.1 µM of each primer, and 1 unit of Taq DNA polymerase. Cycling consisted of denaturation at 95 °C for 5 min; 35 cycles at 94 °C for 30 s, annealing temperature of 65 °C for 30 s, followed by extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The amplification product was subjected to electrophoresis in 1% agarose gel. In carrying out the PCR reaction for SSR16, 50 ng of DNA, 1 unit of Taq DNA polymerase, buffer 1X, 1 mM MgCl₂, 150 µM dNTP, and 0.1 µM of the forward and reverse primers had swelled to 20 µL. The cycling program was denaturation at 94 °C for 2 min, followed by 10 cycles of touchdown PCR decreasing 1 °C at each cycle (from 66 °C to 57 °C), proceeding with another 30 cycles of denaturation at 94 °C, annealing at 57 °C, and extension at 72 °C; 30 s each step. The final extension was at 72 °C for 20 min. The resulting reaction products were separated by electrophoresis in a 6% denaturing polyacrylamide gel and stained with silver nitrate.

2.4.3. Functional Molecular Marker to the CC-NBS-LRR Gene—Locus D

The marker used to analyze Locus D was CARF 005 [12,17]. This marker amplifies a region of the DNA corresponding to the CC-NBS-LRR gene family. Being a dominant marker, it allows for the identification of genotypes D₋ and dd. The controls used were HdT CIFIC 832/2 and Caturra Vermelho CIFIC 19/1, as resistant and susceptible, respectively.

The PCR occurrence had a final volume of 20 µL, containing 50 ng of DNA, 1X PCR occurrence, 2.0 mM MgCl₂, 0.15 mM dNTP, 0.1 µM of each primer, and 1 unit of Taq DNA polymerase. Cycling consisted of denaturation at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, annealing at 60 °C for 30 s, followed by extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

2.4.4. Molecular Marker-Associated Resistance Ck-1 Gene—Locus E

The molecular markers CBD-Sat235 and CBD-Sat207, linked to the *Ck-1* gene that confers resistance to CBD, were used. These markers were identified by [14], validated and mapped by [16] in LG5.

In the analysis of the population with these markers, the HdT UFV 377-15 and UFV 440-10 and the cultivar MGS Catiguá 3 were used as controls carrying the *Ck-1* gene (E₋). The susceptible controls used were Caturra Vermelho CIFIC 19/1 and Catuaí Amarelo IAC 64 (UFV 2148-57) (ee).

PCR amplification was performed in a final volume of 25 µL, containing 50 ng of DNA, 1X PCR reaction buffer, 2.0 mM MgCl₂, 0.1 mM dNTP, 0.4 µM of each primer, and 0.5 units of Taq DNA polymerase. Cycling consisted of denaturation at 95 °C for 5 min, 35 cycles at 94 °C for 45 s, and annealing at 50 °C for 45 s, followed by extension at 72 °C for 45 s and a final extension at 72 °C for 10 min. The products were separated by electrophoresis in a 6% denaturing polyacrylamide gel and visualized by silver nitrate staining [32].

3. Results

3.1. Phenotypic to Races II and XXXIII of *H. vastatrix*

Genetic resistance to CLR was identified in 86% of Central American cultivars, and of these, 83% and 72% were resistant to *H. vastatrix* races II and XXXIII, respectively (Tables S1–S5). In 69% of cultivars, resistance to both races was observed. Cultivars classified as resistant had null LP, IN, SE, UP, and PC, although the IP does not need to be null, since there is no presence of pustules, only symptoms of the disease. The susceptibility of the cultivars to races II and XXXIII was identified in El Salvador and Honduras, and to race XXXIII in cultivars from Guatemala and the Dominican Republic (Tables S3 and S5).

Through mean analysis, significant differences were observed in the parameters IN, SE, UP, and PC, related to resistance for the cultivars that showed susceptibility to race II and XXXIII of *H. vastatrix*. For race II, San Pacho1 and Pacamana demonstrated the lowest means (Figure 2).

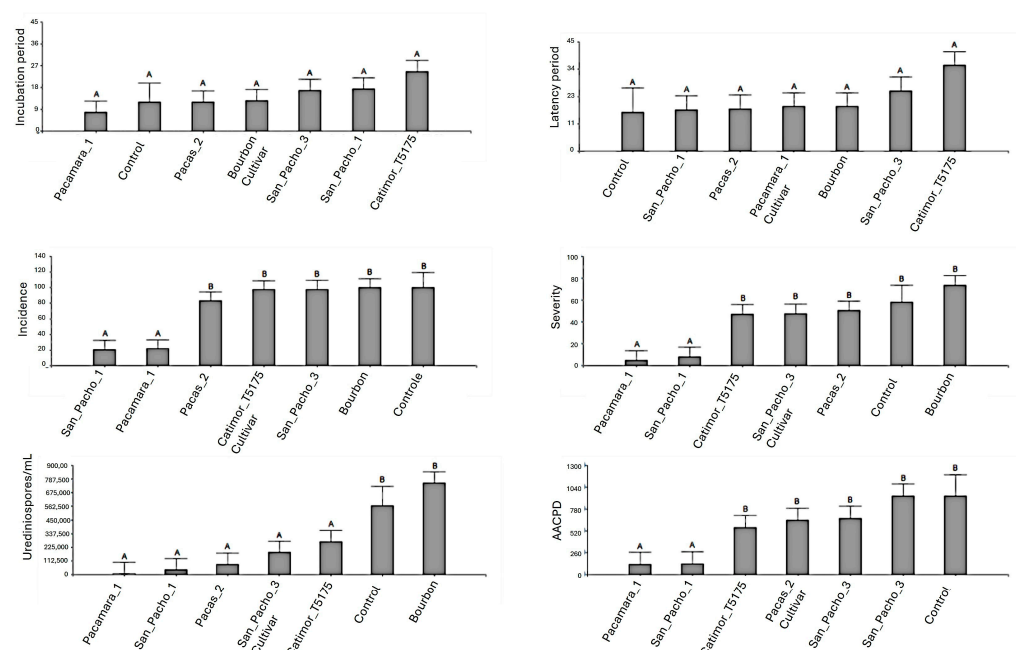


Figure 2. Genetic resistance parameters measured in cultivars susceptible to race II of *H. vastatrix*. Resistance components evaluated, incubation period, latency period, incidence, severity, urediniospores per milliliter, and AACPD—the area under the disease progress curve. Values with different letters show different levels of significance, but those with the same letter mean no significance. Error bars are ± 1 standard deviation of the mean.

The 11 susceptible individuals for race XXXIII had two groups for IN, SE, UP, and three groups for the area under the disease progress curve (AACPD) (Figure 3). Bourbon and the control Caturra showed low means of IP and LP; however, the highest means in the other parameters of genetic resistance for races II and XXXIII. The cultivars with susceptibility to both races analyzed were: Pacamana_1, Pacas_2, San_Pacho_3, Bourbon, and Catimor_T5175.

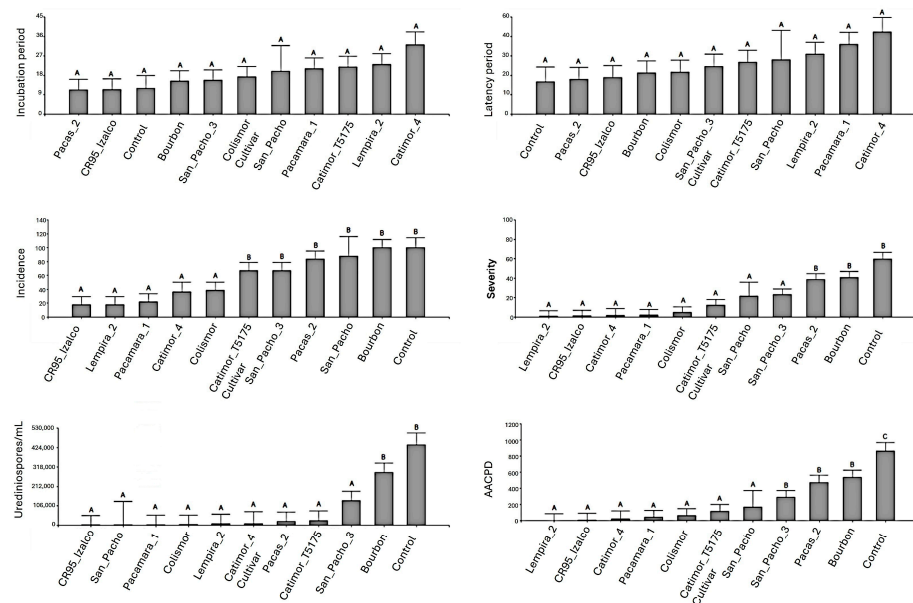


Figure 3. Genetic resistance parameters measured in cultivars susceptible to race XXXIII of *H. vastatrix*. Resistance components evaluated: incubation period, latency period, incidence, severity, urediniospores per milliliter, and AACPD—the area under the disease progress curve. Values with different letters show different levels of significance, but those with the same letter mean no significance. Error bars are ± 1 standard deviation of the mean.

3.2. Genotyping via Molecular Markers for *H. vastatrix* and *C. kahawae*

In the genotyping of the 160 coffee trees, 34 allelic forms of the five loci were identified, with four in Costa Rica (Table S1), 21 allelic forms in El Salvador (Table S2), 14 in Guatemala (Table S3), 18 in Honduras (Table S4), and three in the Dominican Republic (Table S5).

Analysis of the presence of the five loci in homozygous or dominant heterozygous resistance in the genotypes of each country, was carried out by pyramiding the loci ABCDE, BCDE, BDE, and CDE (Figure 4 and Tables S1–S5). Regarding loci B and C, according to [15,16], the presence of one of these loci in dominance is sufficient for there to be resistance to races I, II, and pathotype 001.

Considering the 160 genotypes analyzed, 14 individuals presented the resistance allele of the S_H3 gene (locus A) in a heterozygous manner (8.75%) and 146 genotypes presented it in a homozygous recessive manner (91.25%) (Tables S1–S5). The genotypes that presented the allelic form of the S_H3 gene in a heterozygous manner were: Pacamara 1-5, Pacamara 4-4, Pacas_1-1, Pacas_2-2, and Geisha-3 from the Bourbon–Typica genetic group; Catimor 8-3, 4, and 6, Colismor-1, 2, and 3, IHCAFE 90-5 and 6 from the Catimor genetic group; and San Isidro 48-4 from the Sarchimor group. The genotypes of the Catimor and Sarchimor genetic groups were presented as an exception and possible random crossing since these genetic groups do not carry the S_H3 resistance gene from *C. liberica*.

For the markers of locus B, 44.37% of the individuals presented the resistance allele in dominant homozygosity, 28.75% in heterozygosity, and 26.88% in recessive homozygosity. At locus C, the resistance allele (C_) was presented in 54.29% and the homozygous recessive allele (cc) represented 45.71% of the genotypes. Of the total genotypes, 138 (86.25%) presented at least one dominant locus B or C that can confer resistance to races I, II, or pathotype 001 of *H. vastatrix*. For the resistance gene analog marker (RGA) CC-NBS-LRR, the presence of the locus (D_) that confers resistance to races of *H. vastatrix* was observed in 62.86% of the individuals.

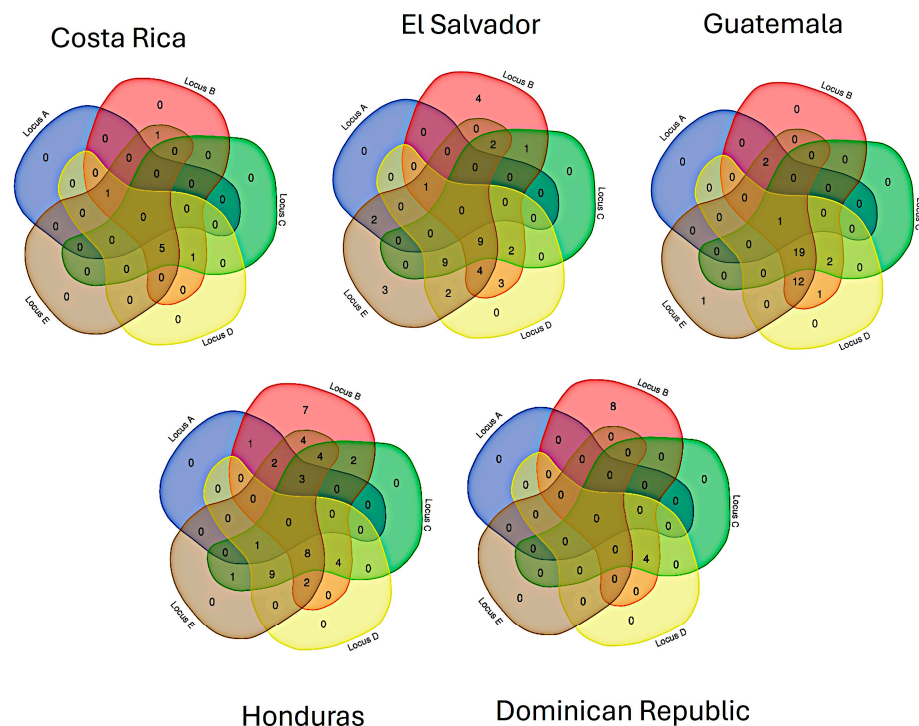


Figure 4. Venn diagrams for classification of Central American coffee in the loci (A–D) for resistance to coffee leaf rust (CLR) and locus (E) for coffee berry disease (CBD). Locus A—blue, Locus B—red, Locus C—green, Locus D—yellow, Locus E—brown. The numbers represent how many genotypes have that set of loci.

In the analysis to determine the presence of the gene that confers resistance to CBD, it was observed that 6.29% of the individuals presented the locus in dominant homozygosis (EE), 60.57% in heterozygosis (Ee), and 33.14% in recessive homozygosis (ee), that is, they do not present resistance to CBD. Considering the loci related to different races of *H. vastatrix*, 89.71% of the individuals presented at least one resistance allele. In total, 32% of the germplasm has the presence of resistance alleles in loci B, C, and D. This grouping of genes in the same individual shows the genetic pyramiding of multiple races.

3.3. Phenotype and Genotype

For race II, it is possible to correlate the presence of the locus with the resistance/susceptibility assessment. According to [15,16], the presence of one of the B or C loci in dominance is sufficient for the genotype to be resistant to races I, II, and pathotype 001. Therefore, it was possible to observe that 138 (86.25%) presented at least one dominant locus B or C that can confer resistance to race II of *H. vastatrix*, and also presented resistance to race II when inoculated with urediniospores of the pathogen (Figure 5 and Tables S1–S5). In addition, it was reported that there were errors in the identification of cultivars, mixtures, or hybridizations in cultivars. In Figure 5, you can observe these allelic variations within each genotype, forming groups A, B, or C. Of the 138 genotypes with confirmed phenotype and resistance loci for race II, 117 (84.78%) genotypes show resistance to race XXXIII when inoculated with urediniospores of the fungus; of these 117 genotypes, 74 (63.4%) presented genes belonging to the three loci (B, C, and D) of resistance to CLR and the locus E to CBD, four genotypes have all loci (A, B, C, D, and E) (Figure 5 and Tables S1–S5).

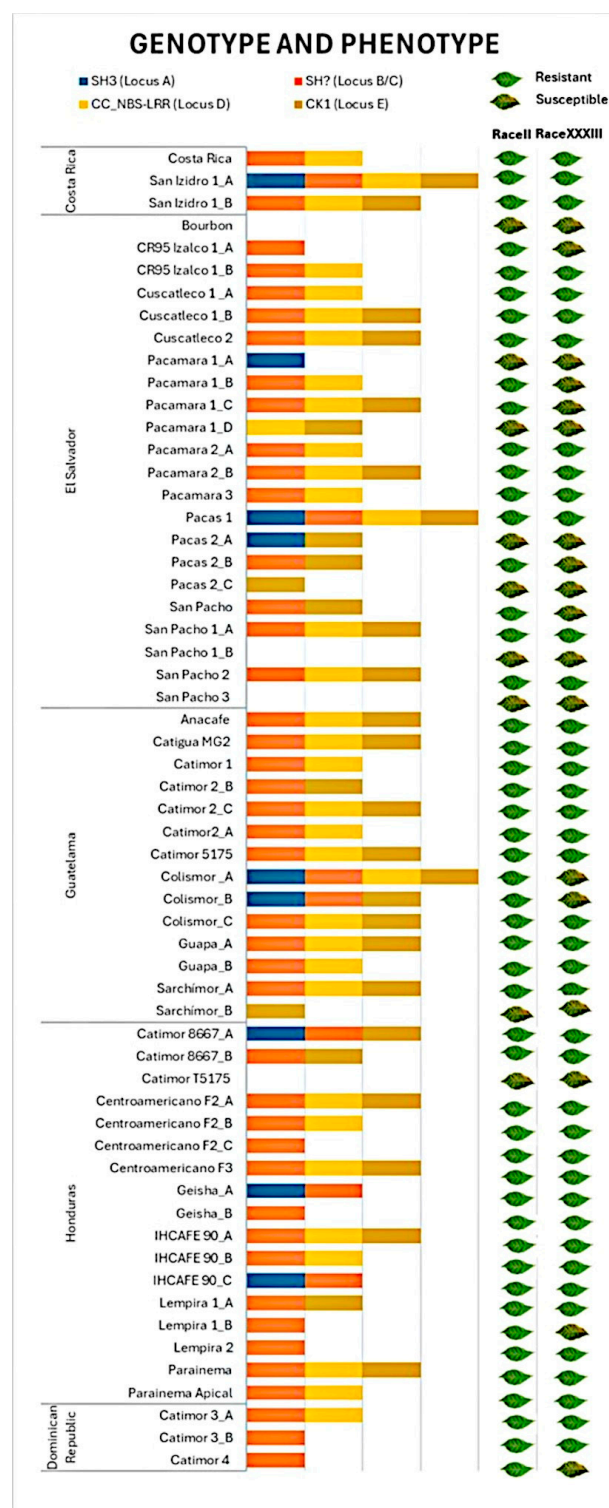


Figure 5. The genotypes and phenotypes of commercial coffee cultivars from America Central. Y-axis: the genotypes of each Central American country are described. On the x-axis, the bars represent the pyramided gene loci: blue bar—the resistance allele of the $SH3$ gene (Locus A); orange bar—the resistance allele of the $SH?$ (unknown gene) located in the locus/QTL of LG 2 and 5 (Locus B/C); yellow bar—the resistance gene analog marker (RGA) $CC-NBS-LRR$ (Locus D); brown bar—the resistance allele of the $Ck-1$ gene located in the locus/QTL (Locus E). Leaves with the presence or absence of resistance for races II and XXXIII.

4. Discussion

Coffee growing in Latin and Central America is predominantly carried out with Arabica coffee cultivars from the genetic group Typica–Bourbon, due to the historical process of the introduction of these plants. Since the 1970s, with epidemics of *H. vastatrix* in America, coffee crops have been in a constant process of renewal with resistant cultivars, and currently as a control against the pandemics of CLR and CBD among producing countries [3]. The use of genetic groups such as Catimor and Sarchimor, originating from crosses with HdT, was chosen due to their multiple resistance to the main pests and pathogens of coffee [34].

Several commercially grown Arabica coffee varieties have been developed through long-term breeding efforts by innovative private institutions, public–private partnerships, and national breeding programs. One example is the Anacafe 14 variety, released in 2014 by the National Coffee Association of Guatemala (ANACAFE). This high-yielding variety is resistant to CLR and drought tolerance. Other varieties, such as Cuscatleco and Parainema, which are resistant to CLR and some *Meloidogyne spp.* nematodes, have been selected by the national breeding programs of Brazil and El Salvador and by IHCAFE in Honduras [35].

The genotypes that showed susceptibility to races II and XXXIII of *H. vastatrix* were analyzed using quantitative resistance parameters, including LP, which is closely related to the multiplication rate of the pathogen, where short periods increase the number of multiplication cycles and, consequently, result in a greater intensity of the disease [25]. Regarding LP, these susceptible cultivars did not show significant differences, leading to maximum periods of up to 34 days being considered.

For IN and SE, there is a direct relationship with UP, where a higher incidence results in greater severity and greater production of urediniospores (Figures 2 and 3). Assessing quantitative resistance often makes it difficult to interpret the information but using the PC parameter makes it possible to estimate the accumulated severity of the disease without having to consider the genotype–environment interaction [36].

The resistance of coffee plants against *Hemileia vastatrix* is governed by at least nine dominant major-effect genes (S_H1 – S_H9), either acting individually or in combination. These genes, known as S_H genes, are responsible for qualitative resistance, providing a high level of defense when present in a homozygous state [8,9]. Quantitative resistance, on the other hand, is associated with minor-effect genes, which contribute to intermediate levels of resistance.

In the interaction between CLR and coffee plants, the pathogen's physiological races attempt to overcome the plant's defense mechanisms. A high resistance reaction is observed when a race lacks the virulence (v) gene required to break the resistance of the corresponding S_H gene, preventing sporulation. However, when a new virulence gene emerges, enabling the race to overcome a specific S_H gene, the plant may still rely on minor-effect genes to mount an intermediate resistance response [8].

This phenomenon may explain the susceptibility of certain cultivars to race XXXIII of *H. vastatrix*. This study showed that many of these cultivars are resistant to race II of *H. vastatrix*, but when inoculated with races that have more complex virulence genes, such as race XXXIII, they tend to be more susceptible, as is the case with CR95 Izalco-1,3, 6; Pacamara 1-1, 2; San Pacho from El Salvador; Anacafé; Colismor-1,2,3 from Guatemala; Lempira 2-1,2,3,4 from Honduras; Catimor 3-4; and Catimor 4-2,3,4 and 5 from the Dominican Republic (Tables S2–S5). These cultivars likely lack the S_H genes required to recognize the corresponding virulence genes $v5$ and $v7$ or $v5$, $v7$, and $v9$ of this race. Instead, they may only carry the S_H5 gene, which recognizes the $v5$ gene associated with race II. Moreover, their susceptibility could be attributed to the absence of minor-effect resistance genes, further reducing their ability to counteract the pathogen. This interplay underscores the

dynamic nature of the host–pathogen interaction, wherein both qualitative and quantitative resistance mechanisms are crucial for plant defense.

Due to the environmental impacts caused by climate change and the constant coevolution of *H. vastatrix*–coffee and *C. kahawai*–coffee, the vulnerability of resistant cultivars has worsened with the emergence of new races of this fungus that are emerging with complex infectious processes capable of overcoming resistance and the appearance of races in regions or countries that previously did not have the disease, such as the Angefa cultivar, which has become susceptible to the pathogen *C. kahawae* due to the emergence of aggressive variants [37,38].

For instance, the Oeiras cultivar, which belongs to the Catimor group, exhibited a high resistance level when released for commercial cultivation in the 1990s. However, after a few years, it lost its qualitative resistance and was reclassified as showing stable resistance. A similar situation occurred with the IPR 100 cultivar, which carries the S_H2 and S_H5 resistance genes. Following the breakdown of the resistance of these two genes in the 1980s, the cultivar’s resistance level decreased to susceptible, similarly to the Catuaí cultivar [8].

Phenotypic and genotypic discrimination correlated with a robust data network, which allowed the characterization of multiple resistances in cultivars/countries. Using molecular markers, homozygous and heterozygous resistance was determined at the studied loci, even in the absence of disease symptoms. Although Arabica coffee is considered autogamous, genotyping allowed the identification of segregation between coffee plants in the same cultivar, which reinforces the theory of a significant percentage of allogamy in the species.

The presence of the S_H3 and *Ck-1* resistance genes in cultivars of the Catimor and Sarchimor genetic groups are valuable discoveries for producers and genetic breeders. S_H3 is acquired by Indian selections, which are tetraploids from natural crosses between *C. arabica* and *C. liberica*, whose gene has been supplanted by the *H. vastatrix* races [39]. The *Ck-1* gene is the most investigated in the scientific community for resistance to CBD, and even without reports in the Americas, this preventive control will be decisive in the event of the possible entry of the pathogen into the continent [37].

Sarchimor has the particularity of presenting more genes for rust resistance; for example, the cultivars Cuscatleco and Parainema have resistance alleles in both QTLs (B_- and C_-) and in the cloned gene (D_-). This fact explains why cultivars derived from Catimor are becoming susceptible to rust and Sarchimor remains resistant. Regarding the CBD resistance locus, Sarchimor has the resistance allele and Catimor does not.

In 8.57% of the population, all loci were identified in recessive homozygosity, namely: all of the genotypes—Bourbon, Catimor T5175, San Pacho 1-4, and San Pacho 3-2 and 3—in which phenotyping showed susceptibility to inoculation with *H. vastatrix*, mainly with race II. The susceptibility to CLR of the Bourbon–Typica genetic group is well described in the literature; however, susceptibility in the Catimor group may be an indication of a resistance breakdown process. Ref. [8] together with CIFC/ISA/UL (Centro de Investigação das Ferrugens do Cafeeiro/Instituto Superior de Agronomia/Universidade de Lisboa) in Portugal, identified five known and two new races in the coffee genotypes, such as Caturra, Typica, and Catimor, cultivated in several Peruvian regions. However, there were exceptions; some genotypes from the Catimor and Sarchimor genetic groups revealed the presence of S_H3 resistance genes, possibly due to random crossing since these genetic groups do not carry the S_H3 resistance gene of *C. liberica*. These results highlight the importance of gene pyramiding to prevent the emergence of new breeds in these Central American countries and in other countries that use materials of the same genetic origin.

Monitoring CLR and CBD across all coffee-producing countries is crucial for overseeing the complex pathosystem involving *H. vastatrix*–coffee and *C. kahawae*–coffee. This

approach enhances disease management efficiency and helps minimize the breakdown of cultivar resistance. In this study, genotypes with both quantitative and qualitative resistance were identified, including the presence of quantitative resistance to races II and XXXIII of *H. vastatrix*, as well as pyramided gene loci (major-effect genes) for resistance to CLR and CBD. Based on these findings, breeders can perform crosses between cultivars with major and minor resistance genes to prevent the quantitative loss of resistance associated with selection for qualitative resistance [8]. Furthermore, the incorporation of the S_H3 gene into breeding programs is a key strategy, as its resistance has proven to be durable in Latin America. The use of these genotypes enables the simultaneous transfer of minor genes, providing intermediate resistance in the event of qualitative resistance breakdown.

5. Conclusions

The results of this study showed gene stacking for resistance to CLR and CBD in genotypes grown in Central America that had the same origin as cultivars used worldwide. In addition to gene stacking, the phenotypic profile of the genotypes for resistance when inoculated with urediniospores to races II and XXXIII of *H. vastatrix* were also shown in this work. The future sustainability of the coffee industry will depend on the ability to develop and distribute genetically improved varieties that combine resistance to pathogens and resilience to climate change, meeting the needs of farmers in specific regions, as well as the expectations of roasters and global consumers. Furthermore, this study revealed errors in passport data or hybridizations in cultivars and even possible resistance breakdown in the Catimor genetic group, in addition to genotypes with the presence of the S_H3 gene, whose resistance has not yet been broken. Therefore, knowledge of the resistance profiles of commercial coffee varieties is essential to the search for strategies in genetic improvement programs or the transition of traditional susceptible varieties, such as Caturra, Typica, and Bourbon, to new resistant varieties with pyramided loci with major- and minor-effect resistance genes, thus hindering future resistance breakdowns by pathogens.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy15010230/s1>, Table S1: Phenotyping and genotyping of cultivars from Costa Rica for *Hemileia vastatrix* and *Colletotrichum kahawae*; Table S2: Phenotyping and genotyping of cultivars from El Salvador for *Hemileia vastatrix* e *Colletotrichum kahawae*; Table S3: Phenotyping and genotyping of cultivars from Guatemala for *Hemileia vastatrix* e *Colletotrichum kahawae*; Table S4: Phenotyping and genotyping of cultivars from Honduras for *Hemileia vastatrix* e *Colletotrichum kahawae*; Table S5: Phenotyping and genotyping of cultivars from the Dominican Republic for *Hemileia vastatrix* e *Colletotrichum kahawae*. Resistance components evaluated: Incubation period (IP), Latent period (LP), Incidence (IN), Severity (SE), Urediniospore production (UP), Area under the disease progress curve (AACPD). R (resistant) and S (susceptible).

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Data Availability Statement: In this manuscript, there are no raw data, the molecular markers used in the analysis were previously available in the literature and are referenced in the manuscript. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The data collected during the performance of this work may be made available by the corresponding author upon reasonable request.

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